Lm = mean linear intercept = Air space enlargement Vvair
Morphometry
tissue loss Vvspt

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Cigarette Smoke Exposure Produces More Evidence of Emphysema in B6C3F1 Mice than in F344 Rats

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Cigarette smoke (CS) causes pulmonary emphysema in humans, but results of previous studies on CS-exposed laboratory animals have been equivocal and have not clearly demonstrated progression of the disease. In this study, morphometry and histopathology were used to assess emphysema in the lungs of B6C3F1 mice and Fischer-344 rats. The animals were exposed, whole-body, to CS at a concentration of 250 mg total particulate matter/m^3 for 6 h/day, 5 days/week, for either 7 or 13 months. Morphometry included measurements of parenchymal air space enlargement (alveolar septa mean linear intercept [L_m], volume density of alveolar air space [V_{vair}]), and tissue loss (volume density of alveolar septa [V_{vspt}]). In addition, centriacinar intra-alveolar inflammatory cells were counted to assess species differences in the type of inflammatory response associated with CS exposure. In mice, many of the morphometric parameters indicating emphysema differed significantly between CS-exposed and control animals. In CS-exposed rats, only some of the parameters differed significantly from control values. The L_m in both CS-exposed mice and rats was increased at 7 and 13 months, indicating an enlargement of parenchymal air spaces, but the V_{vair} was increased significantly only in CS-exposed mice. The V_{vspt} was decreased at both time points in mice, but not in rats, indicating damage to the structural integrity of parenchyma. Morphologic evidence of tissue destruction in the mice included alveoli that were irregular in size and shape and alveoli with multiple foci of septal discontinuities and isolated septal fragments. Morphometric differences in the mice at 13 months were greater than at 7 months, suggesting a progression of the disease. Inflammatory lesions within the lungs of mice contained significantly more neutrophils than those lesions in rats. These results suggest that B6C3F1 mice are more susceptible than F344-rats to the induction of emphysema by this CS exposure regimen and that in mice the emphysema may be progressive. Furthermore, the type of inflammatory response may be a determining factor for species differences in susceptibility to emphysema induction by CS exposure.

Key Words: cigarette smoke; pulmonary emphysema; species differences; mice; rats; morphometry; stereology.

Pulmonary emphysema is the chronic, progressive, and permanent enlargement of air spaces distal to the terminal bronchioles. Emphysema involves destruction of alveolar walls, particularly with regard to lysis of elastin fibers, and results in a reduction of alveolar epithelial surface area (Snider, 1992b; Snider *et al.*, 1985; 1986). Cigarette smoking causes emphysema in humans (Janoff, 1983; Snider, 1992a). Mechanisms of cigarette smoke (CS)-induced emphysema may include pulmonary recruitment of elastase- and other protease-elaborating inflammatory cells, inactivation of lung elastase inhibitors, and disruption of connective tissue repair processes (Campbell, 1986).

The inherited or acquired imbalance between elastase and elastase inhibitors in the pathogenesis of emphysema, the so-called protease/anti-protease model, has been supported by experiments where proteolytic enzymes, including elastases specific to inflammatory cells, have been administered intratracheally to laboratory animals (Gross *et al.*, 1965; Snider *et al.*, 1986). While intratracheal instillation of elastase may produce physiologic and morphologic changes similar to those induced in humans by CS, this model may be limited for evaluating the pathogenesis and response to potential treatment or chemopreventive agents (Campbell, 1986; Thomas and Vigerstad, 1989). Therefore, better models of CS-induced emphysema are needed that more closely mimic the pathogenesis of human disease.

Completely convincing evidence of CS-induced emphysema in animals has been lacking (Thomas and Vigerstad, 1989). This lack of evidence may be due to species and strain differences in susceptibility, the use of experimental CS exposures unlike those in humans (Huber *et al.*, 1981; Snider, 1992b), or failure to apply objective morphometric methods for assessing CS-induced emphysematous change (Thomas and Vigerstad, 1989). Only Huber *et al.* (1981) described rigorous CS exposure protocols that caused morphometrically distinct emphysematous changes in outbred CD rats. This study was limited in that emphysema was assessed after only one 6-month duration of CS exposure, and pulmonary lesions suggestive of low-grade viral or mycoplasmal infections were present in the control animals (Thomas and Vigerstad, 1989). In a study by

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Hoidal and Niewoehner (1983), elastase-induced emphysema in hamsters was potentiated by CS exposure, but CS alone did not induce emphysema in this species. Balb/c mice had focal low-grade emphysema that did not progress in another study of the effects of long-term CS exposure (Keast *et al.*, 1981). Heckman and Dalbey (1982) demonstrated enlargement of alveoli in F344 rats exposed to high concentrations of CS for more than a year, but loss of parenchyma and progression of the air space enlargement were not apparent.

More recently, stronger evidence of CS-induced emphysema in laboratory animals has been produced. Expanded alveolar spaces and alveolar ducts, decreased lung elastin, and physiologic indicators of emphysema were demonstrated in guinea pigs exposed to CS for more than 3 months (Wright and Churg, 1990). Guinea pigs exposed to CS for 6 weeks had enhanced pulmonary macrophage elastase activity and an increase in the parenchyma destructive index (Sansores *et al.*, 1997). Expanded distal air spaces, tissue loss, and decreased lung elastin were demonstrated in Wistar rats exposed to sidestream CS ("passive" smoking) for 3 months (Escolar *et al.*, 1995). Expanded alveolar air spaces and increased alveolar duct areas were demonstrated after 6 months of exposure to CS in the lungs of wild-type, but not transgenic mice deficient in macrophage metalloprotease (Hautamaki *et al.*, 1997). Only Wright and Churg (1990) have shown some evidence that suggests a progression of emphysema in animals exposed to CS. Other animal experiments have not demonstrated a temporal progression, and comparisons between outcomes, based on time, severity, or species, have been confounded by imprecisely documented CS exposure protocols.

In the present study, morphometric parameters of emphysema, including assessments of parenchymal air space expansion and tissue loss, were evaluated in the lungs of two species that had been identically exposed to the same level of CS over the same period of time (7 and 13 months). The results indicate that B6C3F1 mice are more susceptible to the induction of emphysema by CS exposure than F344 rats. Emphysema in these mice may be progressive with longer CS exposure. Further, with respect to the strains of animals used, the two species may differ in the induction of emphysema by CS due to inherent differences in the inflammatory response to lung injury. Because of these differences, the CS-exposed B6C3F1 mouse may be a better model for human emphysema caused by CS.

MATERIALS AND METHODS

Animals and exposures. As part of a series of studies on the effects of combined exposures to pulmonary carcinogens such as CS and X-rays (Finch *et al.*, 1996a), mice and rats were evaluated for the induction of pulmonary emphysema by CS exposure alone. After a 2-week quarantine and acclimatization period, 6–7 week-old female B6C3F1 mice (Charles River Laboratories, Portage, MI) and 6–7 week-old female CDF® F344/CrlBR rats (Charles River Laboratories, Raleigh, NC) were exposed to mainstream CS for 6 h/day, 5 days/week (Monday to Friday), for either 7 or 13 months as described (Chen *et al.*, 1989, 1992; Finch *et al.*, 1995). Briefly, two 70 cm^3 puffs per min from research cigarettes (Type 2R1; Tobacco Health Research Institute, Lexington, KY) were generated by smoking machines (Type 1300; AMESA Electronics, Geneva, Switzerland), diluted with filtered air, and delivered to H-2000 whole-body exposure chambers (Hazleton Systems, Inc., Aberdeen, MD). The mass concentration of CS total particulate material (TPM) was determined by gravimetric analysis of filter samples taken during the exposure periods, and the concentration of CS in the chambers was 250 mg TPM/m^3.

The lungs from 4 mice and 6 rats exposed for either 7 or 13 months were evaluated. Control animals (4 mice for 7 months and 6 mice for 13 months; 6 rats for each time point) were sham-exposed to filtered air. The animals were fed a pelleted ration (Wayne Lab Blox, Allied Mills, Chicago, IL), provided with water *ad libitum*, and maintained under constant temperature and humidity conditions in a 12-h dark/light cycle. Serological analysis of companion animals demonstrated that the mice and rats were free from titers against a panel of common rodent pathogens (Microbiological Associates, Bethesda, MD). In addition, there was no evidence of pneumonia or bronchitis in the control animals.

Necropsy, histology, and stereology. At the end of the exposures, the animals were weighed, then killed by an intraperitoneal injection of pentobarbital and exsanguination through an abdominal aortic incision. The lungs were perfused, via the trachea, with 4% paraformaldehyde at 25 cm of water constant pressure for 6 h. The tracheas were tied off, and the lungs were further fixed for 18 h or more by immersion in 4% paraformaldehyde. After fixation, non-pulmonary tissues were dissected free, and whole lung volumes (V_L) were determined by fluid displacement (Scherle, 1970). The left lungs were systematically sectioned in a dorsoventral-transverse direction at 4-mm intervals with the first slice randomly positioned within the cranial 4 mm of tissue (Bolender *et al.*, 1993). This stratified random sampling generally produced 3 or 4 blocks of tissue from mice and 7 blocks from rats. Tissue blocks were routinely processed and embedded in paraffin. Sections of 5 μm thickness from the caudal faces of the blocks were stained with either hematoxylin and eosin or with alcian blue/hematoxylin and eosin.

The lung sections were evaluated for descriptive histopathology using light microscopy. For stereology, measurements were made on systematically chosen fields in sections of all left lung blocks from mice and in sections of the first, third, fifth, and seventh left lung blocks from rats. Fields were chosen as follows: In mice, at a magnification of 100× (10× objective and 10× ocular), the objective was randomly positioned within the extreme dorsomedial region of a section, and the resultant view was designated as field number 1. Proceeding in a zigzag pattern (i.e., first laterally, then ventrally, then medially, repeatedly), the stage was moved by one-half field diameters across the entire section, and fields were numbered consecutively. One field of the first 3 was randomly selected, and every third field thereafter was evaluated. Once a field was identified, the objective was changed to provide a magnification of 200× prior to creation of a video image. In rats, the same systematic procedure was used, except a magnification of 40× was used to identify fields. The mean number of fields evaluated for mice was 31 (range from 22 to 39; SE = 1.2, n = 18) and for rats was 26 (range from 16 to 34; SE = 1.0, n = 24).

A system for evaluating air space expansion and tissue destruction in vertical anisotropic tissue sections was used (Weibel, 1979; Bolender *et al.*, 1993). Digitized images of the chosen lung fields were generated using a 3-chip CCD camera (Optronics Engineering, Goleta, CA) interfaced with a BH-2 Olympus microscope (20× objective; Olympus Corp., Lake Success, NY), an AG-5 frame grabber card (Scion Corp., Frederick, MD), and a Macintosh Quadra 950 computer (Apple Computer, Inc., CA). Digitized images were captured with NIH Image software (Version 1.59, NIH), and the resulting 640× computer monitor projections were assessed for stereologic parameters with Stereology Toolbox™ software (Morphometrix, Davis, CA). A computer-generated cycloid grid containing 24 evenly spaced points and sine-wave cycloid lines, whose direction was perpendicular to the dorsoventral axis, was placed over the projections. For the measurement of air space expansion, intercepts of alveolar septa with the cycloid lines were counted, and

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TABLE 1
Body Weights of Mice and Rats at Time of Necropsy

	Mice		Rats	
Exposure	7 months	13 months	7 months	13 months
Control	29.8 ± 1.9 (n = 4)	34.5 ± 1.0 (n = 6)	210.7 ± 5.9 (n = 6)	230.6 ± 7.5 (n = 6)
CS	27.3 ± 0.9 (n = 4)	28.3 ± 0.3 (n = 4)	181.5 ± 5.1 (n = 6)	197.5 ± 5.7 (n = 6)

Note. Body wt, g. Values are means ± SE. Two-way ANOVA showed statistically significant effects of exposure and duration ($p < 0.05$), but no effect of an interaction, among CS-exposed and control animals in each species.

a mean linear intercept of alveolar septa (L_m, in μm), the most commonly used morphometric indicator of emphysema (Dunnill, 1962), was calculated:

$$L_m = (\Sigma P_{pa} \times 80\ \mu m) \div \Sigma I_{spt}$$

where ΣP_{pa} is the sum of points of the grid overlying parenchyma, 80 μm is the cycloid length per point, and ΣI_{spt} is the sum of intercepts of the cycloid lines with alveolar septa. Parenchyma was defined as septa and air spaces comprising alveoli and alveolar ducts. The density of the alveolar surface area per unit volume of lung parenchyma ($S_{v_{a,pa}}$, in $\mu m^2/\mu m^3$) and the alveolar surface area for the parenchyma comprising the whole lung (S_a, in cm^2) were also calculated from the intercept data (Weibel, 1979):

$$S_{v_{a,pa}} = (4 \times \Sigma I_{spt}) \div (\Sigma P_{pa} \times 80\ \mu m).$$

$$S_a = (4 \times V_L \times V_{v_{pa}} \times \Sigma I_{spt}) \div (\Sigma P_{pa} \times 80\ \mu m)$$

where $V_{v_{pa}}$ is the percentage of lung volume (V_L) composed of parenchyma (see below) and where there are 2 tissue-air interfaces per intercept.

To assess the loss of tissue, volume densities of lung components were measured by counting points of the grid that overlaid alveolar septa (including alveolar walls juxtaposed to perivascular, peribronchiolar, or subpleural interstitium), alveolar air spaces including those containing inflammatory cells, and other structures such as vessels and airways. The point counts were used to derive the percent volume densities of parenchyma ($V_{v_{pa}}$), alveolar septa ($V_{v_{spt}}$), and alveolar air space ($V_{v_{air}}$) using the following calculations:

$$V_{v_{pa}} = (100 \times [\Sigma P_{spt} + \Sigma P_{air}]) \div \Sigma P_L$$

$$V_{v_{spt}} = (100 \times \Sigma P_{spt}) \div \Sigma P_L$$

$$V_{v_{air}} = (100 \times \Sigma P_{air}) \div \Sigma P_L$$

where ΣP_{spt} is the sum of points overlying alveolar septa, ΣP_{air} is the sum of points overlying alveolar air spaces, and ΣP_L is the sum of points overlying the whole section of lung. Volume densities were also multiplied by V_L for each animal to yield total volume of a particular lung component. Ratios of alveolar surface area (S_a) to septal and parenchymal air space volumes ($V_{v_{spt}} \times V_L$ and $V_{v_{air}} \times V_L$, respectively) were calculated using the following:

$$S_a/(V_{v_{spt}} \times V_L) = (4 \times V_{v_{pa}} \times \Sigma I_{spt}) \div (\Sigma P_{pa} \times 80\ \mu m \times V_{v_{spt}})$$

$$S_a/(V_{v_{air}} \times V_L) = (4 \times V_{v_{pa}} \times \Sigma I_{spt}) \div (\Sigma P_{pa} \times 80\ \mu m \times V_{v_{air}}).$$

For a dimensionless indication of emphysema progression between time points, the percentages of mean control-parameter values for the CS-exposed animals from the 7- and 13-month time points were calculated as follows:

Percent of mean control value =

100 × (parameter value for CS-exposed animal) ÷

(mean value for time-matched control animals).

An index was also devised to assess the type of inflammation present in lung parenchyma of CS-exposed animals. Intra-alveolar inflammatory cells were counted in 6 randomly chosen centriacinar zones of the same left lung sections used for stereology. Terminal bronchioles opening into distinct alveolar ducts were used to identify centriacinar zones. Alveolar duct air spaces in regions extending one-half of a 400× field diameter from the ends of terminal bronchioles and alveolar air spaces immediately adjacent to the alveolar ducts were included for inflammatory cell counts. Intra-alveolar cells were differentiated as either a macrophage or neutrophil; severely degenerated cells that could not be classified were not included in the counts.

Statistics. Body weights and stereologic parameters within each species were analyzed for significant differences by two-way analysis of variance (ANOVA) with exposure and duration as the independent variables. The difference between CS-exposed rats and mice in the number of intra-alveolar neutrophils or macrophages was analyzed for significance by two-way ANOVA with species and duration as the independent variables. In any analysis where a statistically significant interaction was found ($p \leq 0.05$), the Student-Newman-Keuls pairwise comparison method (SNK) was used to isolate which group significantly differed from the others ($p \leq 0.05$).

RESULTS

In this study, mice and rats were exposed to CS from 2R1 cigarettes at exposure chamber concentrations of 250 mg TPM/m^3. At this nominal concentration of TPM, gas concentrations were 170 ppm for carbon monoxide, 70 ppm for total hydrocarbons, and 2.2 ppm for total nitrogen oxides (NO_x). The NO_x consisted of approximately 99% NO and 1% NO_2, and mean exposure concentrations of TPM over the course of the study were within 3% of targeted levels. Previous studies in rats (Finch *et al.*, 1998) have suggested that exposure to this level of TPM is consistent with a heavy human smoking pattern of approximately 3 packs per day.

The CS-exposed animals gained body weight throughout the exposure periods, but their rate of gain was less (data not shown), and the body weights in both species at the end of the exposure periods were significantly less than those of control animals (Table 1). There was a statistically significant effect of the duration of exposure ($p = 0.03$, mice; $p < 0.01$, rats) but

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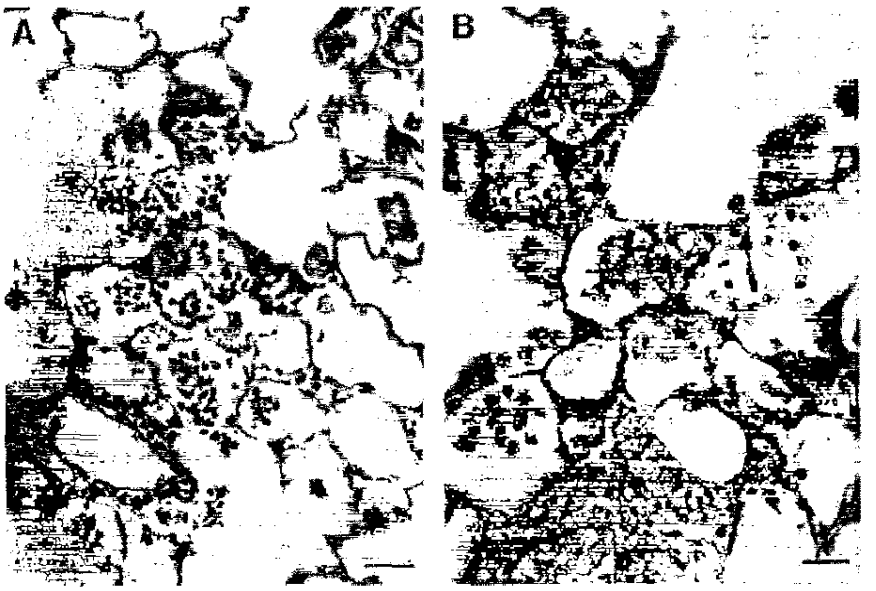

FIG. 1. Inflammation in pulmonary parenchyma of the mouse (A) and rat (B) after 7 months of CS exposure. The inflammatory focus in the mouse (A) contains more neutrophils than the focus in the rat (B). Vacuolated and pigmented macrophages are present (bars = 27 μm).

no interaction between exposure and duration ($p = 0.13$, mice; $p = 0.75$, rats).

In both mice and rats, the CS exposures caused pulmonary inflammation that was characterized by interstitial infiltrates and alveolar exudates of macrophages and neutrophils (Fig. 1). The lesions were multifocally distributed in alveoli, in and around alveolar ducts, and in the alveoli and interstitium adjacent to terminal bronchioles. Some lungs had prominent foci of inflammation in subpleural alveolar air spaces and septa. The infiltrates and exudates sometimes contained tightly aggregated macrophages mixed with variable numbers of neutrophils. The macrophages often had abundant vacuolated tan- or gray-pigmented cytoplasm. Subjectively, neutrophils were more numerous in pulmonary lesions of mice than of rats (Fig. 1). Lesions in the lungs of mice and rats of the 13-month exposure groups were more severe and, in addition to the lesions described, included multifocal perivascular, alveolar septal, and peribronchiolar interstitial infiltrates of lymphocytes and plasma cells mixed with fewer neutrophils and pigmented macrophages. The lungs from the 13-month exposure animals also contained more prominent type-II epithelial hyperplasia associated with some inflammatory foci. Alveolar septal fibrosis was not a feature of lesions in mice. Fibrosis, characterized by slight widening of a few scattered alveolar septa with fibrillar eosinophilic material, was noted only in the lungs of 2 rats exposed to CS for 13 months; the fibrosis was always associated with alveolar epithelial hyperplasia and inflammation.

In general, lungs from CS-exposed mice had diffuse nonuniformity of parenchymal air space sizes characterized by scattered, irregular expansion of alveolar ducts and alveoli (Figs. 2A and 2B). Many of the expanded air spaces were rimmed by blunted septa and distorted alveoli with widened openings. In scattered foci, alveolar septal attenuation and discontinuities were evident, and isolated fragments of alveolar septa were common (Fig. 3). In CS-exposed rats, the air spaces subjectively were no different in regularity from those of the control rats (Figs. 2C and 2D).

The stereologic measurements are listed in Table 2. Two-way ANOVA revealed that the exposure (CS vs. air sham) and the duration of the exposure (7 vs. 13 months) significantly affected several of the parameters in mice. Of particular interest, the L_m was increased and the $S_{Va,pa}$ was decreased in CS-exposed mice relative to control mice. In addition, the mean V_{Vspt} was decreased and the mean V_{Vair} was increased at both time points in CS-exposed mice. There was no statistically significant interaction effect of exposure and duration for any murine parameter. Similarly, the CS-exposed rats had a significantly increased L_m and a significantly decreased $S_{Va,pa}$ at both time points, but, unlike in mice, there was no statistically significant effect of exposure on the V_{Vair} or V_{Vspt}. As with the mice, there was no statistically significant effect of an interaction between exposure and duration for any of these particular parameters in rats.

The CS-associated increase in mouse and rat lung volumes at each exposure duration (Table 2) suggested a compensatory change. There was no significant difference between CS-exposed and control animals in the volume density of lung parenchyma (V_{Vpa}), but when this parameter was multiplied by the lung volume, the total amount of parenchyma ($V_{Vpa} \times V_L$) in CS-exposed animals was larger than that in control animals. The larger amount of parenchyma was attributable to an in-

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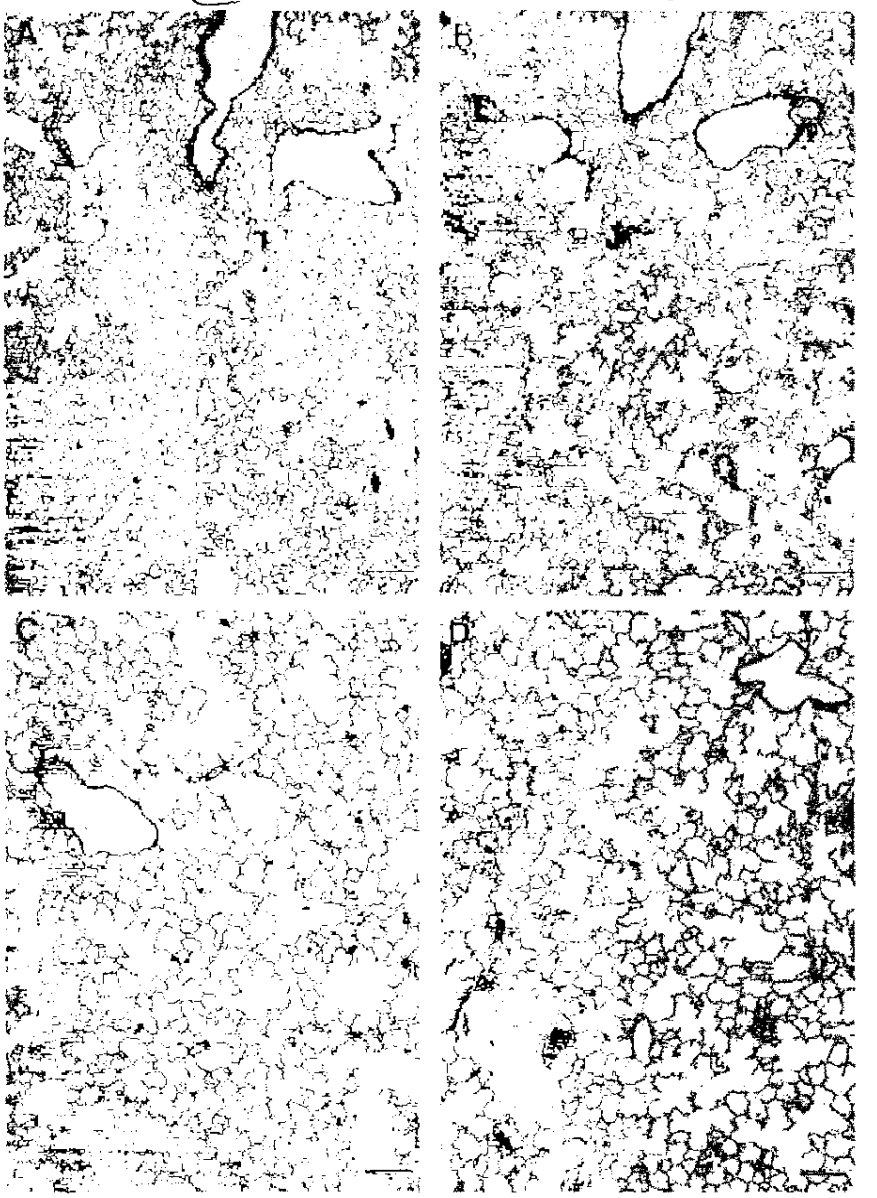

FIG. 2. Morphologic example of emphysema in the mouse after 7 months of CS exposure. In comparison to control (A), there is non-uniform and irregular expansion of air spaces (alveolar and alveolar ductal) causing disorder in the normal parenchymal architecture in the CS-exposed mouse (B). In contrast, when compared to a time-matched control (C), the lung of a rat exposed to CS for 7 months (D) did not have appreciable irregularity or expansion of air spaces (bars = 167 μm).

crease in the total volume of air space ($V_{Vair} \times V_L$) and not to an increase in the total volume of alveolar septa ($V_{Vspt} \times V_L$), even though V_{Vair} was significantly increased only in the CS-exposed mice. The larger total volume of air space in CS-exposed animals with no difference in the total volume of alveolar septa was also reflected by the S_a. There was no statistically significant effect of CS exposure on the S_a from either the mice or the rats, despite the increase in lung volume.

There was no statistically significant difference between CS-exposed and control animals in the surface area to alveolar septa volume ratios ($S_a/[V_{Vspt} \times V_L]$), but, in both mice and rats, the ratio of surface area to alveolar air space volume ($S_a/[V_{Vair} \times V_L]$) was significantly decreased by CS exposure (Table 2). This suggested no significant CS-induced thickening of alveolar septa but a significant CS-induced shape change of the alveolar air spaces with less surface area per volume of air space. In mice, there also was a statistically significant effect of duration on the alveolar surface area to air space volume ratio, and the interaction between the exposure and duration approached the significance level ($p = 0.056$, Table 2). Pair-wise

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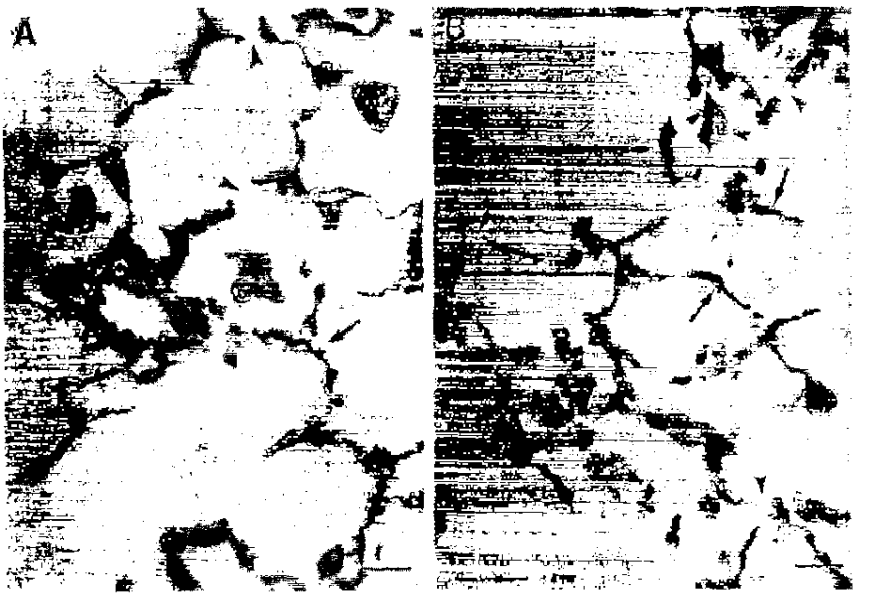

FIG. 3. Air space expansion and alveolar septal destruction in lungs of mice after 13 months of CS exposure. Attenuation and discontinuities (arrowheads) are present in alveolar septa creating isolated septal fragments (arrows). In (A), numerous foamy and pigmented alveolar macrophages are associated with the lesions, while in (B) neutrophils are more prevalent (bars = 17 μm).

analysis (SNK) for this ratio revealed that all mouse exposure-duration groups were significantly different from each other except for the ratios from the 2 CS-exposure groups at 7 and 13 months.

The percent of the mean control values suggested that the emphysema might have worsened with time in CS-exposed mice (Fig. 4). Although two-way ANOVA of the L_m in mice showed no significant effect of an interaction between exposure and duration, the mean percent of control for the L_m in the 13-month exposure group was larger than that of the 7-month group. This suggests that alveolar air space enlargement progressed with the longer exposure. The progressive change in mice was corroborated by a larger percent-volume density of alveolar air space ($V_{V_{air}}$), a larger percent of total air space ($V_{V_{air}} \times V_L$), a smaller volume density

TABLE 2
Stereologic Measurements of Pulmonary Emphysema in Mice and Rats

	B6C3F1 Mice, 7 months		B6C3F1 Mice, 13 months		F344 rats, 7 months		F344 rats,13 months	
Parameter	Control	CS	Control	CS	Control	CS	Control	CS
L_m (μm)	42.2 ± 2.3	51.2 ± 1.6	36.4 ± 0.5	48.3 ± 0.9^{a,b}	57.4 ± 4.1	62.2 ± 2.9	52.2 ± 2.8	63.8 ± 3.3^{a}
$S_{V_{alps}}$ (μm^{-1})	0.096 ± 0.005	0.078 ± 0.002	0.110 ± 0.001	0.083 ± 0.002^{a,b}	0.072 ± 0.005	0.065 ± 0.003	0.078 ± 0.005	0.064 ± 0.003^{a}
V_L (cm^3)	1.14 ± 0.07	1.38 ± 0.06	1.35 ± 0.12	1.76 ± 0.07^{a,b}	6.92 ± 0.33	8.71 ± 0.46	8.87 ± 1.05	10.46 ± 1.09^{a,b}
S_a (cm^2)	982 ± 59	1002 ± 39	1330 ± 107	1353 ± 41^{b}	4885 ± 539	5461 ± 448	6642 ± 683	6274 ± 220^{b}
$V_{V_{ps}}$ (%)	90.9 ± 0.7	93.0 ± 1.0	89.9 ± 1.5	93.1 ± 1.3	97.7 ± 0.9	96.1 ± 0.5	95.2 ± 1.7	94.7 ± 1.7
$V_{V_{ps}}$ (%)	28.6 ± 2.3	24.6 ± 1.1	33.2 ± 1.0	26.1 ± 0.2^{a,b}	20.0 ± 1.2	20.0 ± 1.0	24.6 ± 1.6	20.1 ± 0.9
$V_{V_{air}}$ (%)	62.3 ± 2.5	68.3 ± 2.1	56.7 ± 1.4	67.0 ± 1.5^{a}	77.7 ± 1.2	76.1 ± 1.4	70.6 ± 2.4	74.7 ± 1.7^{b}
$V_{V_{ps}} \times V_L$ (cm^3)	1.03 ± 0.07	1.28 ± 0.06	1.21 ± 0.08	1.63 ± 0.07^{a,b}	6.76 ± 0.34	8.36 ± 0.40	8.46 ± 0.48	9.89 ± 0.37^{a,c}
$V_{V_{ps}} \times V_L$ (cm^3)	0.32 ± 0.03	0.34 ± 0.02	0.45 ± 0.03	0.46 ± 0.02^{b}	1.39 ± 0.11	1.75 ± 0.16	2.21 ± 0.23	2.09 ± 0.11^{b}
$V_{V_{air}} \times V_L$ (cm^3)	0.71 ± 0.06	0.94 ± 0.05	0.76 ± 0.06	1.12 ± 0.06^{a,b}	5.37 ± 0.26	6.61 ± 0.28	6.28 ± 0.33	7.80 ± 0.32^{a,b}
$S_a/[V_{V_{ps}} \times V_L]$ (cm^{-1})	3058 ± 114	2982 ± 208	2986 ± 102	2957 ± 72	3488 ± 121	3138 ± 92	3023 ± 110	3026 ± 138^{b}
$S_a/[V_{V_{air}} \times V_L]$ (cm^{-1})	1407 ± 125	1067 ± 29	1745 ± 26	1153 ± 25^{a,b,*}	905 ± 79	824 ± 49	1059 ± 85	813 ± 48^{a}

Note. All values are means ± SE. See Materials and Methods for definitions. Parameter values within each species were tested for significant differences by two-way ANOVA (exposure×duration). The effect of the interaction between exposure and duration was not significant ($p > 0.05$) for any of the parameters, although the effect of the interaction approached statistical significance for $S_a / [V_{V_{air}} \times V_L]$ in the mice ($*p = 0.056$).

[a] Significant effect of exposure ($p < 0.05$) based on comparison of control values (averaged over 7 and 13 months) with CS-exposed values (also averaged over 7 and 13 months).

[b] Significant effect of duration ($p < 0.05$) based on comparison of 7-month values (averaged over control and CS) with 13-month values (also averaged over control and CS).

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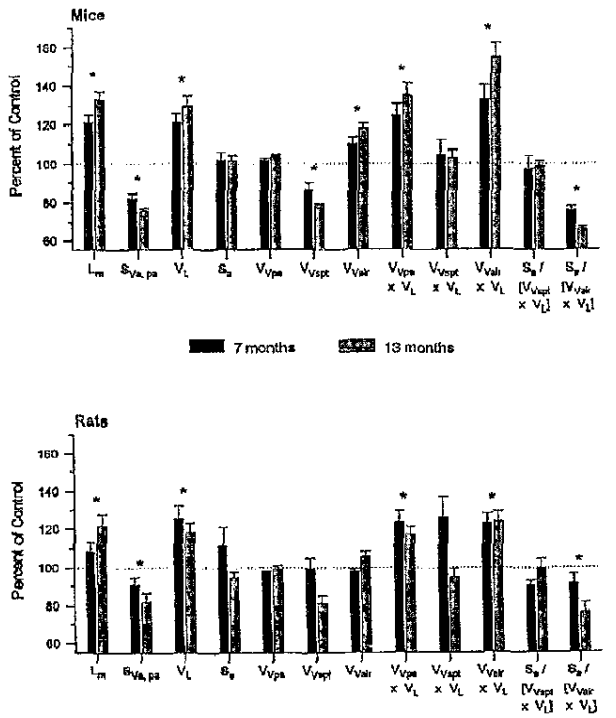

FIG. 4. Percent of control values for stereologic parameters of emphysema in mice and rats exposed to CS for 7 or 13 months. Bars are the mean percentages (plus SE) of the control mean values for CS-exposed individuals. Parameters marked with an asterisk (*) are those that were significantly affected by CS exposure (by 2-way ANOVA, see Table 2). See Materials and Methods for definitions.

of alveolar septa (V_{Vspt}), and a smaller ratio of alveolar surface area to volume of air space ($S_a/[V_{Valr} \times V_L]$) in the 13-month exposure group, in comparison to the respective percent of mean control values in the 7-month group.

The lungs of mice exposed to CS had significantly more neutrophils within centriacinar parenchymal air spaces than the lungs of CS-exposed rats ($p < 0.005$, Table 3). There was no statistically significant effect of the duration of exposure ($p = 0.48$) or interaction between species and duration ($p = 0.20$) on the number of neutrophils. Similar results were obtained by two-way ANOVA of the centriacinar macrophage to neutrophil ratio. The centriacinar inflammatory cell difference between the CS-exposed mice and rats was consistent with the histopathologic findings in the remaining parenchyma (Fig. 1). Subjectively, the lungs of CS-exposed mice had larger numbers of neutrophils in other parenchymal inflammatory foci along with the inflammation in centriacinar regions.

DISCUSSION

The histopathology and stereologic measurements in the present study show that B6C3F1 mice develop morphologic and morphometric features of pulmonary emphysema (Table 2, Figs. 2 and 3) more than F344 rats when exposed to CS at chamber concentrations of 250 mg TPM/m^3 for 7 or 13 months. Moreover, the emphysema appears to be worse in mice exposed to CS for 13 months (Fig. 4). The larger number of neutrophils within the inflammatory lung lesions of the mice, in comparison to those in rats (Fig. 1, Table 3), suggests that infiltrates of neutrophils may be a key factor in the pathogenesis of emphysema in B6C3F1 mice.

Emphysema has long been recognized by pathologists, and several groups have convened to reach consensus definitions of the disease. In the early 1960s, both the World Health Organization (WHO, 1961) and the American Thoracic Society (ATS, 1962) defined human emphysema as enlargement of air spaces distal to the terminal bronchiole, accompanied by destruction of respiratory tissue. Subsequently, in response to a need to review the definition, define tissue destruction, and establish criteria for animal models of emphysema, a workshop was convened by the National Heart, Lung, and Blood Institute (NHLBI) (Snider *et al.*, 1985). The workshop defined human emphysema as "a condition of the lung characterized by abnormal, permanent enlargement of air spaces distal to the terminal bronchiole, accompanied by the destruction of their walls, and without obvious fibrosis." The workshop also defined destruction as "nonuniformity in the pattern of respiratory airspace enlargement so that the orderly appearance of the acinus and its components is disturbed and may be lost" (Snider *et al.*, 1985). Escolar *et al.* (1995) have stated that emphysema in laboratory animals must include evidence of alveolar air space expansion as well as the more problematic demonstration of parenchymal destruction. However, animal models of emphysema were defined less restrictively by the NHLBI workshop to require only qualitative and quantitative stereologic determinations of distal airspace enlargement (Snider *et al.* 1985).

TABLE 3

Comparison between Cigarette Smoke-Exposed Mice and Rats in the Number of Centriacinar Intra-Alveolar Macrophages and Neutrophils

	Number of macrophages	Number of neutrophils[a]	Macrophage:neutrophil ratio[a]
Mice			
7 months	120.0±10.2	74.5±20.8	2.0±0.6
13 months	131.8±11.5	54.8± 1.9	2.4±0.1
Rats			
7 months	130.2± 5.6	26.7± 4.1	5.4±0.7
13 months	140.2±13.9	32.5± 6.5	5.2±1.1

Note. Values are the mean cell counts ± SE from 6 centriacinar zones per animal ($n = 4$ mice or 6 rats per group).

[a] Two-way ANOVA of the number of neutrophils and of the macrophage: neutrophil ratio showed a significant effect of species ($p < 0.005$) but no significant effect of duration or of an interaction between species and duration.

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Although the NHLBI consensus did not require quantitative assessment of destruction in humans or animals, Saetta *et al.* (1985) advocated using a quantitative destructive index to quell the controversy over volume-based morphometric definitions of emphysema. However, Eidelman and coworkers (1990) showed that the destructive index was less effective than the L_m in assessing tissue destruction in a rat emphysema model. Histologic and morphometric data from mice in the present study fulfill criteria of both tissue destruction and air space expansion. Aside from the increased L_m, CS exposure for 7 and 13 months caused attenuation and fragmentation in alveolar septa (Fig. 3) and a decrease in the volume density and surface area density of alveolar septa ($V_{V_{spt}}$ and $S_{V_{a,pa}}$, respectively). This finding indicates a destruction of parenchyma per unit volume of lung (Table 2) in agreement with other assessments of CS-induced parenchyma destruction (Escolar *et al.*, 1995; Huber *et al.*, 1981). The effect of an interaction between the exposure and the duration of exposure was not statistically significant. However, the larger difference in many of the parameters (L_m, $S_{V_{a,pa}}$, $V_{V_{spt}}$, $V_{V_{air}}$, $V_{V_{air}} \times V_L$; Fig. 4) between CS-exposed and control mice of the 13-month group, compared to the differences in the 7-month group, suggested that a longer exposure to CS in mice might cause progression of emphysema.

The CS exposure also induced in rats a statistically significant increase in the L_m, a decrease in the $S_{V_{a,pa}}$, and an increase in the total volume of alveolar air space ($V_{V_{air}} \times V_L$; Table 2). These changes were not as impressive as those in the mice, nor was the trend strong for a worsening of these parameters from 7 to 13 months of CS exposure (Fig. 4). The percentage increase in L_m, for example, was smaller in the rats (108% at 7 months; 122% at 13 months) than the percentage increase in mice (121% at 7 months; 133% at 13 months). Moreover, rat lungs had no statistically significant CS-associated change in the $V_{V_{air}}$ or $V_{V_{spt}}$ parameters. The L_m parameter has been highly correlated with emphysema in human lungs (Verbeken *et al.*, 1992), and on this basis, the CS-exposed rats in the present study could be considered to have emphysema. However, Verbeken *et al.* (1992) have suggested an increase in human lung L_m to greater than 120% of an age-specific expected value should be observed before ascribing significant air space enlargement. With that in mind, the mean L_m value in the rats, unlike the mice, does not really exceed the lower limit of emphysema-associated air space expansion.

The S_a and the total volume of alveolar septa ($V_{V_{spt}} \times V_L$) in CS-exposed mice were not significantly different from those parameters in the control mice, and this may be considered as a lack of morphometric evidence of tissue destruction. On a whole-lung basis, the expansion of the parenchymal air space, as reflected by the increased lung volume and an increased total volume of air space ($V_{V_{air}} \times V_L$; Table 2), mitigated the per unit volume tissue loss parameters of $V_{V_{spt}}$ and $S_{V_{a,pa}}$. This contrasts with the reported hypertrophic lung growth following inhaled toxicant exposure in rodents (Costa *et al.*, 1986; Kodavanti *et al.*, 1998), where air space expansion (increased L_m) has been accompanied by increased S_a. If hypertrophy contributed to the increased lung volumes of the mice in the present study, then the lack of an increase in the S_a suggests concomitant parenchymal destruction. The emphysematous parenchymal destruction shown here (Figs. 2 and 3), with features of "non-uniformity in the pattern of respiratory air space enlargement" (Snider *et al.*, 1985), may have been associated with decreased ventilation of the surface area. Respiratory physiology was not performed on the mice of this study, but physiology results of other attempts at inducing emphysema with CS in laboratory animals have been equivocal (Huber *et al.*, 1981). Snider *et al.* (1986) has suggested that variation in human emphysema physiology creates difficulties for establishing physiologic standards of emphysema in animal models. Thus, physiologic measurements are useful but of secondary importance to morphometry in describing emphysema in laboratory animals.

In both CS-exposed mice and rats, the ratio of alveolar surface area to the volume of alveolar air space ($S_a/[V_{V_{air}} \times V_L]$) was significantly reduced, while the ratio of surface area to the volume of alveolar septa ($S_a/[V_{V_{spt}} \times V_L]$) remained unchanged. The change in the surface area:parenchymal air space volume ratio is associated with the creation of more spherical alveolar air spaces (less surface area for a given volume). Escolar *et al.* (1995) speculated that enhanced alveolar "sphericity" may cause modified gas exchange. Again, the change in the rats was not as severe as that in the mice. The lack of change in the alveolar surface area:septal volume ratio is consistent with an insignificant CS-induced change in the shape of alveolar septa, suggesting that septal shape (e.g., thickness) was not affected by CS exposure in the present study.

A definitive animal model of CS-induced emphysema has been lacking, and the present study is important in attributing morphometric features of emphysema to CS exposure. Another study demonstrated an increase in L_m and a decrease in volume density of parenchyma in Wistar rats exposed for 3 months to sidestream CS, but the progression of the disease was not followed (Escolar *et al.*, 1995). A progressive worsening of physiologic and some morphometric parameters of emphysema (increased $V_{V_{air}}$) was demonstrated in guinea pigs exposed to CS from 3 to 12 months, but a decrease in $V_{V_{spt}}$ was not apparent (Wright and Churg, 1990). Similarly, Heckman and Dalbey (1982) determined that 12 and 18 months of CS exposure to female F344 rats caused an increase in L_m but did not cause a loss in respiratory tissue density ($V_{V_{spt}}$), nor was progressive worsening of the parameters evident. Huber *et al.* (1981) provided morphometric evidence of alveolar air space expansion and tissue loss (increased L_m, decreased $S_{V_{a,pa}}$ and $V_{V_{spt}}$) without an increase in lung volume in CD rats exposed to CS for 6 months, but because lungs were evaluated at only one time point, the authors stated that the changes might not have been permanent or part of a progression. Whether the

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tissue loss represented destruction or CS-mediated inhibition of normal pulmonary development was questioned. However, physiologic measurements in the study that demonstrated loss of pulmonary elastic recoil in CS-exposed animals suggested that the morphologic features were indeed representative of emphysema (Huber *et al.*, 1981).

In the present study, morphometric parameters of emphysema throughout the entire lung were measured using stereology protocols designed to ensure a random sampling of parenchyma (Bolender *et al.*, 1993; Weibel, 1979). Although the protocol did not differentiate changes in centriacinar zones from those in the remaining parenchyma, the CS-induced changes in mice may be representative of panlobular emphysema, as evidenced by the diffusely irregular air space enlargement (Fig. 2). In humans, CS exposure is described as producing centrilobular emphysematous expansion of air spaces centered on respiratory bronchioles which may progress to panlobular lesions (Snider, 1992a). Whether or not the widespread CS-induced emphysema in mice presented here is representative of such a progression is unknown. Other studies in guinea pigs (Wright and Churg, 1990), rats (Escolar *et al.*, 1995), and mice (Hautamaki *et al.*, 1997) have made morphometric distinctions between the expansion of alveolar air spaces and alveolar duct spaces in the induction of emphysema by CS, and all have shown expansion of both components. The CS-induced expansion of alveolar ducts and alveolar air spaces in laboratory animals may not reflect the progression of the disease in humans given the anatomic differences between humans and rodents in the centriacinar region.

In both mice and rats of this study, CS exposure was associated with significantly lower body weights at the ends of the exposure periods. Since severe starvation has been shown to cause morphometric lesions of emphysema in adult animals of a number of species (reviewed by Sahebjami, 1993; Snider *et al.*, 1986), there is a possibility that nutritional inadequacy due to anorexia, malassimilation, or altered metabolism contributed to the emphysema in mice. However, the profound body weight differences in starvation models of emphysema, up to 40% loss in as little as three weeks of feed deprivation (Sahebjami, 1993; Snider *et al.*, 1986), are extreme in comparison to differences in body weights in the present study. The animals in our study did not lose body weight as in starvation models but instead did not gain as much weight or gain weight as rapidly as control animals (data not shown). Although paired-feeding studies would be definitive, it is unlikely that the lower body weights in CS-exposed animals in the present study contributed to any significant morphometric lesions of emphysema.

The difference in pulmonary inflammation between CS-exposed mice and rats (Table 3, Fig. 1) suggests that the preponderance of neutrophils within mouse lungs may predispose this species to the induction of emphysema by CS. The bulk of tissue destruction may have been the result of neutrophilic proteases, and the lesser numbers of neutrophils in the lungs of rats may be the reason why this species was less susceptible to the induction of emphysema by the same level of CS that caused emphysema in mice. Protease and anti-protease activities, however, were not evaluated in the present study. The protease/anti-protease model of emphysema has centered on inherited or acquired deficiencies in serum α_1 protease inhibitor (α_1 PI or α_1 anti-trypsin; Snider, 1992b). Acquired deficiency in α_1 PI may not be completely responsible for CS-induced emphysema. One study has suggested that, while oxidation of α_1 PI by CS may decrease its inhibition of neutrophilic elastase, the overwhelming release of elastase by neutrophils may be the more important factor in determining the elastase/anti-elastase imbalance (Uejima *et al.*, 1995). The larger number of neutrophils within the lungs of mice in the present study supports this hypothesis. Other work has suggested that neutrophilic proteases may be less important than macrophage metalloprotease/elastase (MME) in the induction of emphysema in CS-exposed mice (Hautamaki *et al.*, 1997). Emphysema was not produced in transgenic mice deficient for MME, while wild-type control animals with adequate MME developed emphysema after 6 months of exposure to CS, indicating that MME is essential for the induction of emphysema. However, α_1 PI is degraded by MME (Desrochers *et al.*, 1991; Sires *et al.*, 1994), and thus neutrophil-derived elastase activity may be indirectly augmented by the activity of MME (Hautamaki *et al.*, 1997).

Species differences in proteases or anti-proteases may also influence susceptibility to CS-induced emphysema. Elastase inhibition in mice, for example, is the effect of α_1 PI, while trypsin inhibition is mediated by a different serum protease inhibitor (Martorana *et al.*, 1995). In contrast, human α_1 PI has both elastase- and trypsin-inhibition activities (Janoff, 1983). Further, different strains among a species of laboratory animal may be refractory to the induction of emphysema by CS. Strain A/J mice exposed to CS for 6 months by the same exposure protocol as in the present study (Finch *et al.*, 1996b) did not develop obvious histologic lesions of emphysema. The inflammatory response or the protease/antiprotease characteristics may determine the lack of susceptibility to CS-induced emphysema in this mouse strain. Obviously, differences between species and strains in the protease/anti-protease biochemistry and associated inflammatory responses require further study.

The lack of biochemical analyses of tissue components and proteolytic activities in the present study could be considered a shortcoming in using the B6C3F1 mouse in a model of CS-induced emphysema. Certainly the model would be strengthened if decreased total elastin or increased elastin fragments were demonstrated. Currently, preliminary studies at this Institute indicate that bronchoalveolar lavage fluid from B6C3F1 mice exposed to CS for 30 months, under conditions identical to those used in the present study, contains significantly greater proteolytic activity directed against extracellular matrix components, including elastin (J. Seagrave, unpublished observations). In recognition of the need to further document the type

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of tissue destruction occurring in the B6C3F1 mouse model of CS-induced emphysema, efforts are under way to biochemically characterize alterations at early time points. Coupled with results of the present study, this biochemical analysis may enhance the usefulness of the model in determining the pathogenesis and possible treatment of CS-induced emphysema.

To summarize, the present study is one of few that has shown definitive evidence of pulmonary air space expansion and tissue destruction in animals exposed to CS. This study has shown that B6C3F1 mice were more susceptible than F344 rats are to the induction of pulmonary emphysema by CS exposure. Further, the emphysema appeared to be worse in the mice from the 13-month exposure group. Mice had more neutrophils within pulmonary inflammatory foci than rats, which suggested that the species difference in the inflammatory response might be partially the reason for the difference in emphysema induction by the same CS-exposure regimen. Further study is necessary to elucidate the biochemical mechanisms behind this species difference, in order to better compare responses in laboratory animals to the progression of the disease in humans.

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